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(54) Title: OXIDATION RESISTANT VARIANTS OF PARATHYROID HORMONE

(57) Abstract

Described herein are methionine-substituted variants of parathyroid hormone which, relative to their natural counterparts, exhibit reduced sensitivity to oxidation and substantial PTH activity. Their production using recombinant DNA-based techniques is also described, as is their therapeutic use, e.g. in the treatment of osteoporosis.

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OXIDATION RESISTANT VARIANTS OF PARATHYROID HORMONE

FIELD OF THE INVENTION

This invention relates to variants of parathyroid hormone, t the production of such variants particularly via recombinant DNA technology, and to pharmaceutical compositions containing such variants.

BACKGROUND TO THE INVENTION

Parathyroid hormone, or PTH, is a protein product of mammalian parathyroid glands that elicits various biological effects. Of considerable current interest is the role of PTH as a mediator of the physiologically normal, bone remodelling process. In this regard, PTH acts on osseous tissue to modulate depositi n of skeletal calcium, and thus can cause an increase in bone mass. It has been suggested that the administration of PTH, or agonistic analogues thereof, would be useful therapeutically to treat and/or prevent bone-related disorders, such as osteoporosis.

It has also been revealed that PTH exerts a vasorelaxant effect on the cardiovascular system, and may be useful in controlling systemic blood pressure (see for example the articl by Tenner et al in Vascular Neuroeffector Mechanisms, 4th International Symposium, Raven Press, 1983, pp289-293). It has more recently been revealed that PTH is capable also of modulating the growth of keratinocytes, and may be useful therapeutically in the treatment of skin-related disorders such as psoriasis (s e WO89/03873).

Various forms of PTH, including bovine and human PTH and their analogues, thus hold great promise as therapeutics in the treatment and/or prevention f a number f human disorders. And, curr nt indications ar that th supply of PTH in amounts suffi ient f r

its clinical and commercial applicati ns can be met by exploiting r combinant DNA t chnology for its production. It has been report d that th bacterial host E. coli, for exampl , is capabl f expressing DNA coding for human PTH, to produc a bi active PTH product (see Rabbani t al, J. Biol. Chem., 1988, 263(3):1307). Production of bioactive PTH in the yeast Saccharomyces cerevisia has also been reported (see Gautvik et al, WO88/03165).

A drawback to its value as a pharmaceutical product, however, is that PTH is sensitive to oxidation, to the extent that its biological activity can be eliminated following exposure to certain strong oxidants, especially over time. The oxidative inactivation of PTH can thus be problematical during various stages in its production and purification, can reduce its shelf-life and may reduce half-life and/or bioavailability of the protein *in vivo*. It would thus be desirable to provide PTH variants having a reduced sensitivity to oxidation.

The sensitivity of PTH to oxidation was first appreciated during attempts to label the protein using an oxidative iodination process. As reported in J. Biol. Chem., 1976, 251(1):159, Rosenblatt et al found that a fragment of bovine PTH i.e. bovine PTH(1-34), was inactivated when treated under iodinating conditions. However, when the two methionine residues located at positions 8 and 18 in the peptide were replaced with an isosterically similar but synthetic amino acid, norleucine, they found that activity of the resulting bovine PTH analogue was substantially unaffected by the iodination process. Although the substituted methionines and their norleucine replacements were close structural homologues, however, the norleucine-containing analogue exhibited a significantly reduced bioactivity, of less than about 50% of its methionine-containing counterpart.

The use of a synthetic amino acid such as norleucine as a methi nine r placement pr sents several disadvantages. N rl ucine-

c ntaining analogues of PTH can be produced nly by the labori us route of solution- or solid-phas peptide synthesis. That norleucin is not naturally occurring als rais s th possibilitiy that PTH analogu s containing it may stimulat an immun respons following administration. Moreover, the reduction in bioactivity caused by replacing methionine with norleucine can be undesirable in a pharmaceutical context, particularly since further reductions in bioactivity are likely to occur during storage, and *in vivo*. It would accordingly be desirable to provide variants of parathyroid hormone that exhibit both substantial PTH activity and reduced sensitivity to oxidation. It would be particularly desirabl to provide an alternative method for preparing PTH variants having such characteristics.

It is a general object of the present invention to provide novel variants of parathyroid hormone that exhibit substantial PTH activity and reduced sensitivity to oxidation.

It is another object of the present invention to provid variants of parathyroid hormone having substantial PTH activity and reduced sensitivity to oxidation, which are amenable to production by recombinant DNA technology.

It is also an object of the present invention to provide a pharmaceutically useful composition containing a novel parathyroid hormone variant that exhibits substantial PTH activity and reduced sensitivity to oxidation.

SUMMARY OF THE INVENTION

In the present invention, variants of parathyroid hormone exhibiting substantial PTH activity and reduced sensitivity to oxidati n ar obtained by r placing at least on methionine resident in PTH with a genetically encoded amino acid.

Mor particularly, and according to general aspect of the present invention, ther is provided a parathyroid hormone variant having substantial PTH activity and reduced sensitivity t oxidation, of th formula:

$$[X^{8}Y^{18}]PTH$$

wherein at least one of X and Y is a genetically encoded amin acid other than methionine and cysteine.

According to one embodiment, the parathyroid hormone variants are those in which Y in the above formula is methionine, and X is selected from alanine, valine, leucine, isoleucine, serine and tryptophan.

According to another embodiment, the parathyroid hormone variants are those in which X in the above formula is methionine, and Y is a genetically encoded amino acid other than methionine and cysteine.

According to another embodiment of the present invention, Y in the above formula is other than methionine and cysteine, and X is selected from alanine, valine, leucine, isoleucine, serine and tryptophan.

In using genetically encoded amino acids rather than synthetic amino acids as methionine replacements, the present invention provides PTH variants that in addition to having substantial PTH activity and reduced sensitivity to oxidation, are also amenable to production by recombinant DNA techniques. According to another aspect of the present invention, therefore, there is provided a cellular host having incorporated expressibly therein a DNA molecule which codes for a PTH variant of the present invention. In a related aspect of the present invention, there is provided a method for producing a PTH variant having substantial PTH activity

and a reduced sensitivity to xidation, c mprising th step of culturing a ellular host in which DNA coding for the PTH variant is expressibly incorporated.

The PTH variants of the present invention are suitably employed as therapeutics. According to another aspect of the present invention, therefore, there is provided a pharmaceutical composition comprising a pharmaceutically acceptable carrier and a PTH variant of the present invention.

These and other aspects of the invention are now described with in greater detail and with reference to the accompanying drawings, in which:

BRIEF REFERENCE TO THE DRAWINGS

Figure 1 is a map of plasmid pX in which DNA coding for human PTH is linked operably with DNA enabling expression thereof in E. coli;

Figure 2 provides the nucleotide sequence of the human PTH-encoding region of the plasmid shown in Figure 1, and also provides for reference the amino acid sequence of human PTH. Methionin residues at positions 8 and 18 are highlighted using boxes;

Figure 3 illustrates graphically the relative activities of human PTH and human PTH variants, before oxidation; and

Figure 4 illustrates graphically the relative activities of human PTH and human PTH variants, after oxidation.

DETAILED DESCRIPTION OF THE INVENTION AND ITS PREFERRED EMBODIMENTS

Th present invention relates to PTH variants that exhibit substantial PTH activity and a reduc d sensitivity to oxidati n.

In the present specification, PTH activity is defined in the context of the osteosarcoma-based adenylate cyclase assay employed conventionally in the art. Briefly, this assay provides an *in vitro* determination of the extent to which PTH stimulates adenylate cyclase activity in rat osteosarcoma cells of the 'UMR' lineage, and thus provides an indication of PTH effects on bone tissue *in vivo*. Protocols for conducting the assay have been described by Rodan et al, 1983, J. Clin. Invest., 72:1511 (in which the osteosaracoma cells of the ROS lineage are employed) and by Rabbani et al, 1988, Endocrinol., 123:2709 (which employs the line UMR-106). PTH variants that exhibit, in the UMR-based assay, an EC_{50} of at least 2,000 nM i.e. 2,000 nM or lower, are herein characterized as having "substantial" PTH activity.

In addition to retaining substantial PTH activity, the PTH variants of the present invention are also characterized by a sensitivity to oxidation that is reduced, relative to a methionine-containing PTH counterpart. A PTH variant having a "reduced sensitivity to oxidation" will exhibit, following exposure to an oxidant, an activity as measured in the osteosarcoma-based assay which exceeds the activity exhibited by a similarly treated PTH control. An assay suitable for determining sensitivity to oxidation entails a two step procedure, in which the PTH variant is first exposed to oxidizing conditions, for example using hydrogen peroxide as oxidant, and is then assayed for activity in the osteosarcoma-based assay just described. Protocols suitable for assaying oxidation sensitivity are described by O'Riordan et al, 1974, J. Endocrinol., 63:117, and are outlined in the examples herein. Human PTH variants having a reduced sensitivity to oxidation will exhibit in the osteosarcoma-based assay an activity that is at least greater than a similarly treated human PTH control.

The PTH variants of the present invention conform to the general formula:

$[X^{8}Y^{18}]PTH$

wherein at least on of X and Y is a genetically encoded amino acid other than methionine and cysteine. In the above formula, th numerals identify the location of the amino acids X and Y within the PTH molecule, relative to the N-terminal amino acid thereof. For consistency and as is conventional in the art, X and Y are assigned the same positional number when present in the context of N-terminally truncated or extended forms of PTH, such as anal gues or fragments of PTH. In the formula, the term PTH refers to any genetically encodable form of PTH in which a methionine is resident at one or both of positions 8 and 18. Such forms include, but ar not limited to, porcine PTH which has a single methionine resident at position 8, as well as human PTH and bovine PTH, both of which have methionines resident at position 8 and position 18. The term PTH also embraces rat PTH, which has a methionine residue at positions 8, 21 and 41, and chicken PTH which has a methi nin residue at positions 5, 8, 26 and 80.

The term "human PTH" refers to the mature form of the horm n , which consists of 84 amino acids arranged in the sequence reported by Kimura et al, 1983, Biochem. Biophys. Res. Comm., 114(2):493. The terms "human PTH", "hPTH" and "hPTH(1-84)" are used interchangeably herein.

The terms "bovine PTH", "rat PTH" and "porcine PTH" refer also to the mature form of the hormone, each of which consists of 84 amino acids arranged in the sequences reported by Keutmann et al in Current Research on Calcium Regulating Hormones, Cooper, C.W.(Ed.), 1987, University of Texas Press, Austin, pp.57-63.

While the term PTH refers unless otherwise stated to th mature form of a given mammalian PTH species, it will be appr ciated that the strategy h rein described can be applied also to genetically encodable, methionin -containing analogues and

fragments of PTH which exhibit PTH activity, in ord r to generate variants there f exhibiting r duc d sensitivity to xidation. The term "PTH analogu " is used h rein with reference t methi nine-containing forms of PTH having an altered amino acid sequence, such as an amin acid substitution at a non-methionine site. The term "PTH fragment" is used herein with reference to methionine-containing peptides having PTH activity and comprising at least the first 27 amino acids of PTH, and more desirably the first 34 amino acids of PTH.

As will be appreciated from the examples provided herein, the oxidation sensitivity exhibited by PTH can be reduced by replacing at least one methionine resident in PTH with virtually any genetically encoded amino acid. Of course, cysteine is an oxidizable amino acid, and should not be used as a methionin replacement. For PTH species in which methionines are resident at two positions, such as human PTH and bovine PTH, it has been found that the amino acid used as a methionine replacement must be carefully selected in order to preserve substantial PTH activity. Furthermore, the site at which replacement is effected also affects both oxidation sensitivity and PTH activity of the resulting variant.

More particularly, it has been found that sensitivity to oxidation is reduced and the PTH activity substantially preserved, when the methionine at position 18 is replaced with virtually any genetically encoded amino acid. Accordingly, there is provided according to one embodiment of the present invention a parathyroid hormone variant of the formula:

$$[Y^{18}]PTH$$

wherein Y is a genetically encoded amino acid other than methionin and cysteine. Desirably, Y is selected from among the group c nsisting of alanin , valin , leucin , is leucine, serine and

tryptophan. Most suitably, Y is selected from among the group consisting of alanine, valine, leucine and isoleucine. Preferably, Y is leucine.

Specific compounds conforming to the formula [Y^{18}]PTH include human PTH variants such as [Leu^{18}]hPTH, [Ile^{18}]hPTH, [Ala^{18}]hPTH, and [Val^{18}]hPTH, as well as bovine PTH equivalents thereof.

Replacement of the methionine at position 8 of PTH has a more significant effect on both oxidation sensitivity and PTH activity than does replacement of the methionine at position 18. Relative to replacement of Met18, a greater reduction in sensitivity to oxidation is realized when Met8 is replaced. However, replacement of Met^8 can also cause a significant decline in PTH activity, and the amino acid chosen to replace Met^8 must be selected carefully. According to one embodiment of the present invention, there are provided PTH variants which conform to the formula:

[X^8]PTH

wherein X is an amino acid selected from among the group consisting of alanine, valine, leucine, isoleucine, serine and tryptophan. Desirably, X is selected from alanine, valine, leucine and isoleucine. Most suitably, X is selected from valine, leucine and isoleucine. Preferably, X is leucine.

Specific compounds conforming to the above formula include human PTH variants, such as [Leu^8]hPTH, [Ile^8]hPTH and [Val^8]hPTH, as well as bovine PTH and porcine PTH equivalents thereof.

In addition to PTH variants characterized by single site methionine replacement i.e. conforming to one of the formula [X^8]PTH and [Y^{18}]PTH, the present invention provides PTH variants in which methionines resident at both positions 8 and 18 are replaced, of the formula:

$[X^8Y^{18}]PTH$

wh rein X and Y are independently s lected, Y is a genetically encoded amino acid other than m thi nin and cyst ine, and X i an amino acid selected from among the group c nsisting of alanine, valine, leucine, isoleucine, serine and tryptophan. Relative to variants characterized by single site methionine replacement, PTH variants conforming to the above formula are virtually resistant to oxidation. With selection of X from the amino acid group recited above, substantial PTH activity is also retained.

Specific PTH variants conforming to the above formula, i.e., having two-site methionine replacement, include human PTH variants such as $[Leu^8Leu^{18}]$hPTH, $[Ile^8Leu^{18}]$hPTH, $[Val^8Leu^{18}]$hPTH, $[Ser^8Leu^{18}]$hPTH, $[Ala^8Leu^{18}]$hPTH, $[Trp^8Leu^{18}]$hPTH, $[Leu^8Ile^{18}]$hPTH, $[Leu^8Val^{18}]$hPTH and $[Ile^8Ile^{18}]$hPTH and bovine PTH equivalents thereof.

The PTH variants of the present invention are genetically encodable proteins, and may therefore be produced either by chemical synthesis or, more desirably, using recombinant DNA-based production techniques. The solid phase peptide synthesis technique has been successfully applied in the production of human PTH and can be used for the production of the PTH variants of the present invention (for guidance, see Kimura et al, supra, and see Fairwell et al, Biochem., 1983, 22:2691). Success with producing human PTH on a relatively large scale has been reported by Goud et al in J. Bone Min. Res., 1991, 6(8):781, incorporated herein by reference. This production approach generally entails the use of automated synthesizers and appropriate resin as solid phase, to which is attached the C-terminal amino acid of the desired PTH variant. Extension of the peptide in the N-terminal direction is then achieved by successively coupling a suitably protected form of the next desired amino acid, using either FMOC- or BOC-based chemical pr tocols typically, until synthesis is complete. Pr t cting

groups ar then cleaved from the peptide, usually simultaneously with cl avage of p ptide from the resin, and the peptide is th n isolat d and purified using conventional t chniques. Such pr cedures are generally described in numer us publications and reference may be made, for example, to Stewart and Young, Solid Phase Peptide Synthesis, 2nd Edition, 1984, Pierce Chemical Company, Rockford, Illinois.

More desirably, and in accordance with one aspect of the present invention, the PTH variants are produced by culturing a cellular host in which DNA coding for the desired PTH variant is expressibly incorporated. Incorporation of the desired DNA, in expressible form, can be achieved using the now conventi nal recombinant DNA-based approach, wherein DNA coding for the PTH variant is linked operably with DNA enabling expression of the PTH variant-encoding DNA, to form a recombinant DNA expression construct which is then introduced into the selected cellular host by DNA-mediated transformation, electroporation or the lik . A cellular host having DNA coding for a PTH variant incorporated "expressibly" therein is characterized by the ability to yield the desired expression product, when cultured appropriately. A cellular host having DNA coding for a PTH variant incorporated "stably" is able to retain such DNA during culturing, and to transmit such DNA to its progeny through at least several generations. For eucaryotic cellular hosts, such stability is typically conferred by genomic integration of the PTH variant-encoding DNA. In bacteria, which typically harbour transforming DNA in the form of autonomously replicating plasmids, such stability is usually ensured by culturing a strain carrying plasmid-conferred antibotic resistance in the presence of the antibiotic.

For expression in the cellular host, DNA coding for a selected PTH variant may be obtained using techniqu s that are well stablish d in the art. F r exampl , a DNA sequence coding for a

given PTH variant may be synthesized *de novo* in accordance with methods standard in the gen synthesis art. Briefly, this entails the succ ssive 3′ t 5′ c upling f suitably prot cted nucleotide r agents in an automat d synth sizer such as th Applied Biosy tems Inc. model 380B DNA synthesizer, and then the recovery by g l purification of the deprotected polynucleotide. The block ligation approach may be employed, whereby "blocks" of oligonucleotide pairs, up to about 80 nucleotides in length, are prepared and ligated in correct succession by overhang complementarity, as described for example by Wosnick et al in Gene, 1989, 76:153. In an alternative approach, the desired DNA may be synthesized *in toto*, and then amplified by polymerase chain reaction (PCR), using the approach described by Barnett et al in Nucl. Acids Res., 1990, 18(10):3094.

It will be appreciated that alternative strategies may also be applied to generate DNA coding for the desired PTH variant. For instance, DNA coding for human PTH may be obtained and then used as a template e.g. mutagenized site-specifically, to introduce the desired amino acid change at the genetic level. DNA coding for human PTH may be obtained from an appropriate human cDNA library, from a commercial source or by *de novo* synthesis according to the procedures outlined above, and in accordance with the PTH-encoding nucleotide sequence reported for example by Hendy et al, Proc. Natl. Acad. Sci. USA, 1981, 78:7365, incorporated herein by reference, or a PTH-encoding equivalent thereof. The PTH-encoding DNA template may be converted to DNA coding for a PTH variant using the well established oligonucleotide-directed mutagenesis technique, as generally described for example by Kunkel et al, 1985, Proc. Natl. Acad. Sci. USA, 82:488. This technique is conveniently accomplished with high efficiency using the E. coli-based system for synthesis and propogation of the altered gene in an appropriate vector, such as M13mp18. Kits useful for performing such procedures *in vitro* are available commercially. Also suitabl for obtaining PTH variant-encoding DNA from a PTH-encoding templat

is the related, technique in which site-directed mutagenesis is achieved using a PCR-based approach. One variant of this method, termed "recombinant PCR" is described by Higuchi et al, Nucl. Acids. Res., 1988, 16:7351, and a modified "megaprimer" PCR approach is described in Biotechniques, 1990, 8(1):404.

Once obtained, DNA coding for the desired PTH variant is incorporated stably and expressibly into a cellular host selected to serve in production of the PTH variant. A variety of organisms are suitable as hosts for production of the PTH variants. These include eukaryotic hosts including yeasts such as Saccharomyces, Pichia and Kluveromyces, filamentous fungus hosts including Aspergillus species such as nidulans, niger (or awamori) and oryzae, insect cell hosts, and mammalian cell hosts including the CHO and COS cell lines. The PTH variants are not dependent on glycosylation for activity, and thus can suitably be produced in bacterial hosts including Streptomyces, Bacillus and, preferably, in E. coli. Recombinant DNA expression systems and culturing media/protocols enabling production in these hosts of a desired protein have already been established, and these systems may be employed in the conventional manner for the specific purpose of producing PTH variants. E. coli production of PTH variants may be achieved, for example, using expression systems based on the lac promoter (see Rabbani et al, Biochem., 1990, 29:10080) and expression/secretion systems based on the tac promoter (see Wong et al, EP 357,391). Yeast expression may be achieved using expression systems based for example on the expression controlling regions of the alpha-1 mating factor gene as described by Gautvik et al in WO88/03165. Production in Aspergillus may be achieved using secretion systems based on expression controlling regions of the A. nidulans alcA gene or the A. niger glucoamylase gene, as described for example by Gwynne et al in WO86/06097.

The PTH variant produced upon culturing of the production host is extracted and purified using techniques that are also

established in the art. In general, the human PTH variants have characteristics that are similar generically to those exhibited by human PTH, and may therefore be extracted and purified in substantially th same manner. Like PTH, the variants have a net positive charge at neutral pH (pI of about 9.3) and can be purified therefore by ion exchange chromatography, e.g. using cation exchange columns. The PTH variants are also, like PTH, hydrophobic in nature, and may therefore be purified by hydrophobic interaction chromatography e.g. on columns having a phenyl-Sepharose matrix. Also, of course, molecular sieves may be used to separate PTH variants from other proteins unrelated by size, and affinity columns may be employed which comprise PTH affinity agents such as hydroxyapatite or PTH antibody. Preferably, purification of the PTH variant is achieved by applying the protein mixture to a cation exchange column e.g. S-Sepharose, and then applying the eluted retentate to a column having a hydrophobic matrix e.g. a column having a phenyl, octyl or butyl side chain such as phenyl-Sepharose, phenyl-Superose, octyl-Sepharose or butyl 650M. The retentate eluted from the hydrophobic matrix is then subjected to final purification using reversed phase high performance liquid chromatography (HPLC).

While the oxidation sensitivity of human PTH typically demands that great care be taken during purification to guard against oxidative inactivation, such as the use of anti-oxidants e.g. cysteine or B-mercaptoethanol, and the use of cold temperatures to slow the oxidative process, purification of the PTH variants of the invention requires less stringent control during purification and handling. For example, anti-oxidants are not required during purification of human PTH variants in which both Met^{8} and Met^{18} are replaced, but may be employed if desired.

For therapeutic use, a PTH variant is desirably purified to the extent that it migrates as a single peak on reversed phase HPLC, and exhibits a single band on polyacrylamid gel

electroph resis in the presence of SDS. Once purified, the PTH variant may be formulated to provid pharmaceutical compositions suitable for treating the vari us clinical conditions for which PTH replacement therapy is indicat d. Compositions containing PTH variant may, for example, be delivered systemically to treat bone disorders such as osteoporosis and cardiovascular conditions, and for these purposes are suitably formulated either as injectables r ingestibles or for nasal insufflation. Sterile injectabl compositions are preferred, and will generally comprise an effective dose of the PTH variant, in admixutre with normal salin and suitable solubilizing agent e.g. dilute acetic acid. The PTH variant may alternatively be applied topically, as a cream, lotion, ointment or as an aerosol, to treat psoriasis and related skin disorders. A suitable cream will comprise an effective dose of the PTH variant, in combination with carriers of standard composition e.g. in a triglyceride base.

The dose of PTH variant effective to treat a given clinical condition will depend of course on the nature and severity f the condition, and on such other factors as are normally considered and evaluated in clinical trials and by the attending physician. For treating osteoporosis, the PTH variant is administred in amounts large enough to stimulate bone remodelling, but not so large as to cause net bone resorption or sustained increase in serum calcium levels. Reference may be made to US patent 4,698,328 for guidance on the administration of PTH to treat osteoporosis. Using th effective PTH doses in a given clinical situation for guidance, the dose of PTH variant required to elicit a similar effect can be calculated based on the relative activity of the PTH variant. For example, [Leu^{18}]hPTH(1-84), [Leu^{8}]hPTH(1-84) and hPTH(1-84) are substantially equipotent, and effective doses of these PTH variants are thus similar to those of hPTH. It is expected that the gr ater oxidative stability of the PTH variants will provide for extended *in vivo* half-life, and thus somewhat small r doses may b used r simular d ses may be administered less frequently.

Like PTH, the PTH variants may be administered in combination with other agents useful in treating a given clinical condition. When treating osteoporosis and other bone-related disorders for example, the PTH variants may be administered in conjunction with a dietary calcium supplement or with a vitamin D analogue (see US 4,698,328). Alternatively, the PTH variant may be administered, preferably using a cyclic therapeutic regimen, in combination with bisphosphonates,as described for example in US 4,761,406, or in combination with one or more bone therapeutic agents such as calcitonin and estrogen.

Examples

The examples which follow describe production of PTH and PTH variants. Production of these proteins was achieved using, as a matter of convenience only, an E. coli-based system substantially as described by Wong and Sutherland in European patent application 89308753.6 (published as EP357,391 on 7 March 1990), the contents of which are incorporated herein by reference. This system makes use of the commonly available E. coli JM101 strain as host and employs as vector a pUC18 derivative, designated pX. As is shown in Figure 1, pX incorporates the par element of pSC101 to enhance frequency of plasmid transmission, the lacIq gene of pMMB22 to enable overproduction of the lac repressor, and a PTH-excretion cassette. Incorporated in the excretion cassette is human PTH-encoding DNA that was synthesized using the block ligation technique reported by Wosnick et al, supra, and in accordance with the PTH-encoding nucleotide sequence reported by Hendy et al, supra. Fused 5′ of, and precisely to, the PTH-encoding DNA is the signal sequence of the E. coli ompA gene, which is capable of directing the PTH portion of the expression product across the host inner membrane, and ultimately to the culturing medium. For regulated expression of the coding region, the plasmid operably incorporates the tac promoter, the lac operator and a consensus ribosomal binding site. Transcriptional termination is controlled by the E. coli trpA gene terminator, and translational stop codons

are provided in all thre reading frames, immediately 3' of the PTH-encoding DNA.

Thus, the pX expression v ct r, used for the production of human PTH and PTH variants, is substantially the same as that described by Wong and Sutherland, supra, except that the multiple cloning site downstream of the PTH gene contains cleavage sites f r the restriction enzymes ClaI, BamHI, XbaI, StuI and PstI, in th order indicated on Figure 1. The precise nucleotide sequence f the PTH-encoding region of the excretion cassette is illustrat d in Figure 2. The plasmid pX may thus be contructed by incorporating into pUC18 at any suitable site therein (1) an excretion cassette having the functional components described by Wong et al, *supra;* (2) the $lacI^q$ gene from pMMB22, and (3) the par element excised from pSC101.

Example 1 - Production of human PTH(1-84)

Plasmid pX was transformed into competent E. coli JM101 using standard procedures. Positive transformants were indentifi d following growth overnight at 30°C on plates containing 2YT/agar and 70µg/ml ampicillin. PTH-producing transformants were then examined for PTH activity, following growth in shake flasks, by IRMA analysis of conditioned medium, and frozen stocks of the selected transformants were subsequently prepared by mixing an equal volume of the shake flask culture with sterile glycerol t yield 50%(v/v) glycerol stocks. These stocks were subsequently stored at -80°C. When needed, transformants were recovered from the frozen stock by scraping, and were then streaked on ampicillin-containing plates of 2YT/agar.

To produce human PTH, freshly plated transformants were picked as single col nies and then in culated into 50ml Erlenmeyer flasks containing 15ml of a liquid medium which contained 2YT, glucose and

ampicillin in the standard mixture. Following overnight growth with shaking at 30°C, the cultures were diluted 20-fold with fresh medium, and then grown for thr e hours at 30 C with shaking. Expression of th PTH-encoding DNA was then de-repressed by additi n of 1.0mM IPTG. Aft r growth for four hours in the presence of IPTG, the culture was cooled to 4°C and centrifuged. The supernatant was then harvested and human PTH contained therein was recovered and assayed for PTH activity.

To obtain sufficient quantities of human PTH(1-84) and the PTH variants for purification and bioassay, larger volumes of conditioned media were collected. In particular, freshly plated transformants were picked as single colonies and then inoculated into 500ml flasks containing 200ml of the medium described above. Following overnight growth with shaking at 30°C, the cultures were inoculated into 2L bioreactors containing 1.5L of the liquid medium, and then grown for 5 hours at 30°C with stirring. Expression of the PTH- or PTH variant-encoding DNA was then induced by addition of I.0mM IPTG. After growth for 3-4 hours in the presence of IPTG, the culture was cooled to 4°C and centrifuged. The supernatant was then harvested, and the PTH or PTH variant contained therein was purified in the manner described in Example 7.

The examples which follow describe production of PTH variants. To obtain DNA coding for these variants, the *in vitro* site-directed mutagenesis technique described by Kunkel et al, supra was applied. To perform this procedure there was first obtained plasmid RX which is an M13mp18-based plasmid lacking a functional tac promoter. Plasmid RX thus served as the template for conducting mutagenesis on the PTH-encoding DNA, in order to generate DNA coding for a desired PTH variant. The particular mutagenesis strategy is described in the examples below.

Example 2 - Production of a [Leu^{18}] variant of PTH

To provide DNA coding for a PTH variant in which Met^{18} is replaced by leucine, plasmid RX was first recovered in single stranded form and about 1µg thereof was incubated, at 85°C in Hin buffer, with about 100ng of a mutagenic oligonucleotide capable of hybridizing specifically to that region of the PTH gene containing the Met18 codon. The specific sequence of the oligonucleotide, designated M2, is shown below where underlining indicates the codon change relative to the PTH-encoding template:

M2 oligo: 5′ CTCTCTCCAGCGAGTTC 3′
template: 3′.....GAGAGAGGTAGCTCAAG..... 5′

After slow cooling, the annealed fragment was treated with DNA polymerase I (Klenow) in the presence of all four dNTPs, for about 2 hours at 37°C and then for 4 hours at room temperature, in order to form the full length double-stranded plasmid, designated pRXM2. Competent host JM101 was then transformed by pRXM2, and plaques were screened by restriction digest analysis and by DNA sequencing to select those carrying the desired mutation.

pRXM2 is then digested with NruI and XbaI and the resulting small fragment is isolated by low melting point agarose. Plasmid pX is similarly digested, and the large NruI/XbaI fragment is isolated. The relevant isolated fragments are then ligated, to form plasmid pXM2, which carries DNA coding for [Leu^{18}]hPTH. This was confirmed by restriction digest analysis and DNA sequencing.

Competent E. coli JM101 was transformed with pXM2 and the transformants were then selected in accordance with the procedures outlined in Example 1. Supernatant containing the [Leu^{18}]PTH for subsequent purification was then obtained by culturing the pXM2 transformant, in the manner described by example 1.

Example 3 - Production of a [Leu^8] variant of PTH

In a manner similar to that described in Example 2, there was obtained DNA coding for a human PTH variant in which Met^8 is replaced by leucine. In particular, single stranded pRX was incubated with an oligonucleotide having the sequence provided below, where underlining indicates the codon change relative to the PTH-encoding template:

M1 oligo: 5′ CCAGGTTATG<u>CAG</u>AAGCTGTATTTCAC 3′
template: 3′ .....GGTCCAATACGTATTCGACATAAAGTG..... 5′

A double stranded plasmid carrying the Leu8 codon, designated pRXM1, is then cut with NruI/XbaI and the isolated small fragment is ligated with the large fragment of NruI/XbaI-digested pX. E. coli was then transformed by the resulting plasmid pXM1, and the transformant was cultured in the manner outlined in Example 1 to yield supernatant containing [Leu^8]hPTH.

Example 4 - Production of a [Leu^{8}Leu^{18}] variant of PTH

In a manner similar to that described in Example 3, there was obtained DNA coding for a human PTH variant in which both Met^8 and Met^{18} are replaced by leucine. This was achieved by incubating pRXM2, which already bears the Leu^{18} codon, with the M1 oligo (example 3) which introduces the Leu^8 codon, to yield plasmid pRXC1. Following sequencing which confirmed incorporation of the Leu^8 and Leu^{18} codons, the NruI/XbaI fragment was cloned as described in example 2, and the resulting plasmid was transformed into E. coli JM101. Transformants were selected, the selected transformants were grown in shake flasks, and the shake flask supernatants containing [Leu^{8}Leu^{18}]hPTH were recovered and stored frozen for subsequent analysis, all in accordance with the methods described in Example 1.

Example 5 - Production of an [Ala^8] variant of PTH

In a manner similar to that described in Example 3, there was obtained DNA coding for a human PTH variant in which the Met^8 codon was replaced by an alanine codon. In particular, the Met^8 codon in pRX was replaced site-specifically using an oligonucleotide having the sequence shown below, where underlining identifies the introduced codon change:

M3 oligo: 5′ CCCAGGTTATGAGCAAGCTGTATTTCAC 3′
template: 3′ .....GGGTCCAATACGTATTCGACATAAAGTG..... 5′

This generated plasmid pRXM3, the small NruI/XbaI fragment of which is ligated to the large NruI/XbaI fragment of pX to yield pXM3. E. coli JM101 was then transformed with pXM3, and the transformants were cultured to provide supernatants containing [Ala^8]hPTH, in the manner described in Example 1.

Example 6 - Production of additional PTH variants

In the manner substantially as described above in example 4, supernatants containing additional PTH variants were obtained by culturing E. coli transformants habouring PTH variant-encoding DNA, as listed below. In each case, the DNA coding for [Leu^{18}]hPTH (Example 2) was used as template and was altered at the Met^8 codon using the noted oligonucleotide (underlining is used to indicate the replacement codon):

i) [Ile^{8}Leu^{18}]hPTH; using an oligonucleotide having the sequence 5′-CAGGTTATGGATAAGCTGTATTTC-3′;

ii) [Asp^{8}Leu^{18}]hPTH; using an oligonucleotide having the sequence 5′-CAGGTTATGGTCAAGCTGTATTTC-3′;

iii) [Asn^{8}Leu^{18}]hPTH; using an oligonucleotide having the sequence

5′-CAGGTTATGGTTAAGCTGTATTTC-3′;

iv) [Tyr^{6}Leu^{18}]hPTH; using an oligonucle tide having the sequence 5′-CAGGTTATGGTAAAGCTGTATTTC-3′; and

v) [Arg^{6}Leu^{18}]hPTH; using an oligonucleotide having the sequenc 5′-CAGGTTATGACGAAGCTGTATTTC-3′;

Also obtained by mutagenizing the [Leu^{18}]PTH-encoding template (example 2), was DNA coding for the following additional PTH variants:

vi) [Val^{6}Leu^{18}]hPTH; where Val is encoded by GTT
vii) [Ser^{6}Leu^{18}]hPTH; where Ser is encoded by TCG
viii) [Ala^{6}Leu^{18}]hPTH; where Ala is encoded by GCG
xi) [Trp^{6}Leu^{18}]hPTH; where Trp is encoded by TGG
x) [Gln^{6}Leu^{18}]hPTH; where Gln is encoded by CAG
xi) [Glu^{6}Leu^{18}]hPTH; where Glu is encoded by GAA
xii) [Gly^{6}Leu^{18}]hPTH; where Gly is encoded by GGG
xiii) [Lys^{6}Leu^{18}]hPTH; where Lys is encoded by AAG

E. coli transformants were obtained and cultured, and supernatants containing the variant were collected individually as described in example 1, for analysis as now described in example 7.

Example 7 - Purification and Evaluation of PTH and PTH variants

The conditioned medium collected from the transformants of Examples 1-6 was, in each case, adjusted to about pH 4 with glacial acetic acid. In some but not all cases, mercaptoethanol was then added to a final concentration of 10mM and the solution was centrifuged. It was found that mercaptoethanol was unnecessary in the PTH variant purification process. The supernatant was harvested and then passed through a column containing the cation exchang resin S-Sepharose FastFlow (Pharmacia, bed volume 50ml) pr -

equilibrated with 0.04M ammonium acetat /10mM B-mercapto thanol (pH4.0). PTH or the PTH variant, bound to th resin, was luted by applying a concentrati n gradient of amm nium acetat as eluant of from 0.04M - 1.0M ammonium acetate/10mM B-mercapto thanol (pH4.0). PTH or the PTH variant eluted from the resin at about 0.6M ammonium acetate. Eluant fractions, containing PTH or the PTH variant (as measured by the Allegro two-site IRMA purchased form Joldan Diagnostics, California, catalogue #40-2170, or by absorbance at 280nm), were combined to provide PTH or the PTH variant at about 60-70% purity.

Samples of greater purity were obtained by subjecting th combined fractions to a chromatographic separation using the resin phenyl-Sepharose FastFlow (Pharmacia). More particularly, the pH of the combined S-Sepharose fractions was adjusted to pH 8 with 5N NaOH. This solution was then applied to a column containing phenyl-Sepharose (6ml bed volume), pre-equilibrated with the buff r (6 volumes of 1.0M ammonium acetate (pH4.0) and 4 volumes of 40mM ammonium acetate (pH4.0), then adjusted to pH 8.0 with 5N NaOH). PTH or the PTH variant, adsorbed to the column, was then eluted using as eluant a concentration gradient of buffer to 0.6M ammonium acetate (pH8.0).

Fractions containing PTH activity (as measured by Allegro two-site IRMA or monitored by A_{280}) were combined and then desalted by passage through a cartridge containing reversed phase C-18 resin e.g. Sep-Pak (Waters Inc.) or Amberchrom CG71 resin (Toso Haas) pre-equilibrated with 0.1% TFA. PTH or the PTH variant bound to the resin was eluted with 0.1% TFA/80% acetronitrile. The desalt d preparations were then frozen in liquid nitrogen, lyophilized and stored at -20°C.

Thawed or fresh samples of human PTH(1-84) and of PTH variants obtained as described abov wer then valuated f r biological

activity in a UMR-106 based adenylat cyclase assay and the protocol as described by Rabbini et al, 1988, Endocrinology, 123:2709, which is incorporated herein by referenc . As noted, rat osteosarcoma cells of th UMR line are stimulated by PTH to produce adenylat cyclase, an enzyme which catalyzes intrac llular conversion of ATP to its cylic monophosphate analogue, cAMP. In this assay therefore, PTH activity is determined by assaying radiometrically the formation of cAMP in PTH-stimulated UMR cells. Also assayed for comparison was a sample of synthetic human PTH, purchased from Bachem Inc. (Torrence, California - catalogue #PCAL 175). The results of the assays, expressed in terms of EC_{50} (concentration of PTH or variant effective for half-maximal stimulation of adenylate cyclase activity), are presented in Table 1.

TABLE 1 - Relative activities of PTH variants

PTH variant	EC_{50} (nM)
human PTH	1.0
[Leu^{18}]	1.5
[Leu^{8}]	5.5
[$Leu^{8}Leu^{18}$]	5.7
[$Leu^{8}Leu^{18}Tyr^{34}$]	22
[$Ile^{8}Leu^{18}$]	32
[$Val^{8}Leu^{18}$]	90
[$Ala^{8}Leu^{18}$]	850
[Ala^{8}]	1200
[$Ser^{8}Leu^{18}$]	1400
[$Trp^{8}Leu^{18}$]	1900
[$Asn^{8}Leu^{18}$]	>5000
[$Gln^{8}Leu^{18}$]	>5000
[$Asp^{8}Leu^{18}$]	>5000
[$Glu^{8}Leu^{18}$]	>5000
[$Lys^{8}Leu^{18}$]	>5000
[$Arg^{8}Leu^{18}$]	>5000
[$Tyr^{8}Leu^{18}$]	>5000
[$Gly^{8}Leu^{18}$]	>5000

In reference to Table 1, it will be not d that substantial PTH activity is exhibited by only certain methionine-substitut d PTH

acid substitutions, additions or deletions have been introduced. Analogues of the PTH variants of the invention thus represent additional embodiments of the present invention.

Analogues of the PTH variants specifically contemplated herein include those having amino acids replaced at positions other than 8 and 18. Analogues of this class include those in which the phenylalanine at position 34 is replaced a tyrosine residue or other amino acid residue which is receptive to radiolabel conjugation. Specific such analogues include [$Leu^{18}Tyr^{34}$]hPTH, [$Leu^{8}Tyr^{34}$]hPTH, [$Ile^{8}Leu^{18}Tyr^{34}$]hPTH and [$Leu^{8}Leu^{18}Tyr^{34}$]hPTH. To produce such analogues, DNA coding for a PTH variant is site-specifically mutagenized to effect the desired amino acid replacement at the genetic level, and then expressed in a microbial host in the manner exemplified in example 9 hereinbelow.

Also within the scope of the present invention are C-terminally truncated analogues of the methionine-substituted PTH variants herein described. Analogues of this class include N-terminal fragments of the PTH variants, which consist of at least the first 27 N-terminal residues. Specific such analogues include those comprising the first 34 N-terminal residues, such as [Leu^{18}]hPTH(1-34), [Leu^{8}]hPTH(1-34), [$Leu^{8}Leu^{18}$]hPTH(1-34), and Tyr^{34} analogues thereof. It is reasonably expected that in addition to reduced oxidation sensitivity, these fragments will exhibit a biological activity comparable to their full length counterparts, as measured in the osteosarcoma assay. Accordingly, these fragments may be used in a manner similar to human PTH(1-84). The N-terminal fragments may also be produced by recombinant DNA approach, using PTH variant-encoding DNA that has been mutagenized site specifically to introduce a translational stop codon immediately downstream of the residue 34 codon.

sampl is dissolv d in ass y m dium and appropriat diluti ns established, for use in the bioassay. Bioactivity of the oxidant-expos d samples was then assess d in the UMR-based adenylate cyclase assay. The results are presented graphically in Figures 3 and 4, which show the relative activities of human PTH and human PTH variants in the absence of oxidant (but using a mock oxidation treatment i.e. incubation in the absence of hydrogen peroxide for 8 minutes at room temperature in 0.1M acetic acid) (Figure 3) and after oxidation i.e. incubation in the presence of 1.5% (v/v) hydrogen peroxide for 8 minutes at room temperature in 0.1M acetic acid (Figure 4).

In reference to Figures 3 and 4, it will be noted that th replacement of methionine with a genetically encoded amino acid has the effect of reducing the sensitivity to oxidation exhibit d by PTH, regardless of whether the replacement is effected at position 8 alone, at position 18 alone or at both positions. It will be noted as well as that replacement of methionine at position 8 al n results in a significantly greater reduction in oxidation sensitivity than does replacement at position 18 alone. The variant [Leu^{8}]hPTH(1-84) is about sixty-fold more bioactive than hPTH(1-84) under these conditions, whereas the variant [Leu^{18}]hPTH(1-84) is about two-fold more active than hPTH(1-84). The greatest reduction in sensitivity to oxidation is seen when methionines at both positions 8 and 18 in PTH(1-84) are replaced. The variants [Leu^{8}Leu^{18}]hPTH and [Leu^{8}Leu^{18}Tyr^{34}]hPTH exhibit a bioactivity under these conditions that is about 400-fold greater than hPTH(1-84).

Taken together, these results demonstrate that the sensitivity to oxidation exhibited by PTH is markedly reduced when one or both of its resident methionines is replaced by a genetically encoded amin a id other than methi nine. It is contemplated that this enhanced oxidative stability will b exhibited als by analogues of the methionine-substituted PTH variants, in which on or more amino

acid substitutions, additions or deletions have been introduced. Analogues of the PTH variants of the invention thus represent additional embodiments of the present invention.

Analogues of the PTH variants specifically contemplated herein include those having amino acids replaced at positions other than 8 and 18. Analogues of this class include those in which the phenylalanine at position 34 is replaced a tyrosine residue or other amino acid residue which is receptive to radiolabel conjugation. Specific such analogues include [$Leu^{18}Tyr^{34}$]hPTH, [$Leu^{8}Tyr^{34}$]hPTH, [$Ile^{8}Leu^{18}Tyr^{34}$]hPTH and [$Leu^{8}Leu^{18}Tyr^{34}$]hPTH. To produce such analogues, DNA coding for a PTH variant is site-specifically mutagenized to effect the desired amino acid replacement at the genetic level, and then expressed in a microbial host in the manner exemplified in example 9 hereinbelow.

Also within the scope of the present invention are C-terminally truncated analogues of the methionine-substituted PTH variants herein described. Analogues of this class include N-terminal fragments of the PTH variants, which consist of at least the first 27 N-terminal residues. Specific such analogues include those comprising the first 34 N-terminal residues, such as [Leu^{18}]hPTH(1-34), [Leu^{8}]hPTH(1-34), [$Leu^{8}Leu^{18}$]hPTH(1-34), and Tyr^{34} analogues thereof. It is reasonably expected that in addition to reduced oxidation sensitivity, these fragments will exhibit a biological activity comparable to their full length counterparts, as measured in the osteosarcoma assay. Accordingly, these fragments may be used in a manner similar to human PTH(1-84). The N-terminal fragments may also be produced by recombinant DNA approach, using PTH variant-encoding DNA that has been mutagenized site specifically to introduce a translational stop codon immediately downstream of the residue 34 codon.

Example 9 - Production of a Tyr^{34} analogue of [Leu^{8}Leu^{18}]hPTH(1-84)

Using pRXC1 which contains DNA coding for [Leu^{8}Leu^{18}]hPTH(1-84) as a template (see Example 4), the oligonucleotide-directed mutagenesis technique was performed as described in example 2 replace the Phe^{34} codon with a tyrosine codon. The change was effected using an oligonucleotide having the sequence;

5'-CTCCAAGGGCAACGTAATTGTGCACATCC-3'

The resulting plasmid pRXC2 is then cut with NruI and BamHI and the small fragment is ligated to the large fragment of similarly cut pX, thereby generating pXC2. Transformants harbouring pXC2 were then cultured, and [Leu^{8}Leu^{18}Tyr^{34}]hPTH(1-84) contained in the conditioned medium was purified and assayed as described in Example 7. The results are included in Table 1 and Figures 3 and 4, and show that the variant exhibits reduced sensitivity to oxidation and retains substantial PTH activity. By introducing the tyrosine residue, there is provided a substrate amenable to radiolabelling, such as with I^{123} in the manner reported in US 4,409,141, and which is useful for imaging studies and for *in vitro* binding analyses.

WE CLAIM:

1. A parathyroid hormone variant having substantial PTH activity and a reduced sensitivity to oxidation, of the formula:

$$[X^{8}Y^{18}]PTH$$

wherein at least one of X and Y is a genetically encoded amino acid other than methionine and cysteine.

2. A parathyroid hormone variant according to claim 1, wherein Y is methionine.

3. A parathyroid hormone variant according to claim 2, wherein X is selected from among the group consisting of alanine, valine, leucine, isoleucine, serine and tryptophan.

4. A parathyroid hormone variant according to claim 2, wherein X is selected from valine, leucine and isoleucine.

5. A parathryoid hormone variant according to claim 2, which is $[Leu^{8}]PTH$.

6. A parathyroid hormone variant according to claim 1, wherein X is methionine.

7. A parathyroid hormone variant according to claim 6, wherein Y is selected from among the group consisting of alanine, valine, leucine, isoleucine, serine and tryptophan.

8. A parathyroid hormone variant according to claim 7, wherein Y is selected from alanine, valine, leucine and isoleucine.

9. A parathyroid hormon variant according t claim 8, which is $[Leu^{18}]PTH$.

10. A parathyroid hormone variant according to claim 1, wherein X and Y are both genetically encoded amino acids other than methionine and cysteine.

11. A parathyroid hormone variant according to claim 10, wherein X is selected from among the group consisting of alanine, valine, leucine, isoleucine, serine and tryptophan.

12. A parathryoid hormone variant according to claim 11, wherein X and Y are the same.

13. A parathryoid hormone variant according to claim 12, which is [$Leu^{8}Leu^{18}$]PTH.

14. A parathyroid hormone variant according to claim 11, wherein X and Y are different.

15. A parathyroid hormone variant according to claim 14, wherein Y is leucine.

16. A parathyroid hormone variant according to claim 14, which is selected from [$Ile^{8}Leu^{18}$]PTH, [$Val^{8}Leu^{18}$]PTH, [$Ser^{8}Leu^{18}$]PTH, [$Ala^{8}Leu^{18}$]PTH and [$Trp^{8}Leu^{18}$]PTH.

17. A parathyroid hormone variant according to any preceding claim, which is a variant of human parathyroid hormone.

18. A fragment of a parathyroid hormone variant according to claim 17, said fragment having substantial PTH activity and reduced sensitivity to oxidation.

19. An analogue of a parathyroid hormone variant according to claim 17 or claim 18, said analogue having substantial PTH activity and a reduced sensitivity to oxidation.

20. An analogu of a parathyroid h rmone fragment according to claim 18, wherein Phe^{34} is r placed by tyrosine.

21. An analogu of a parathyroid hormone fragment according to claim 20, which is [$Leu^{8}Leu^{18}Tyr^{34}$]PTH.

22. A cellular host having incorporated expressibly therein a DNA molecule which codes for a parathyroid hormone variant as defined in any preceding claim.

23. A cellular host having incorporated expressibly therein a DNA molecule which codes for a parathyroid hormone variant as defined in claim 17.

24. A cellular host according to claim 23, wherein said host is E. coli.

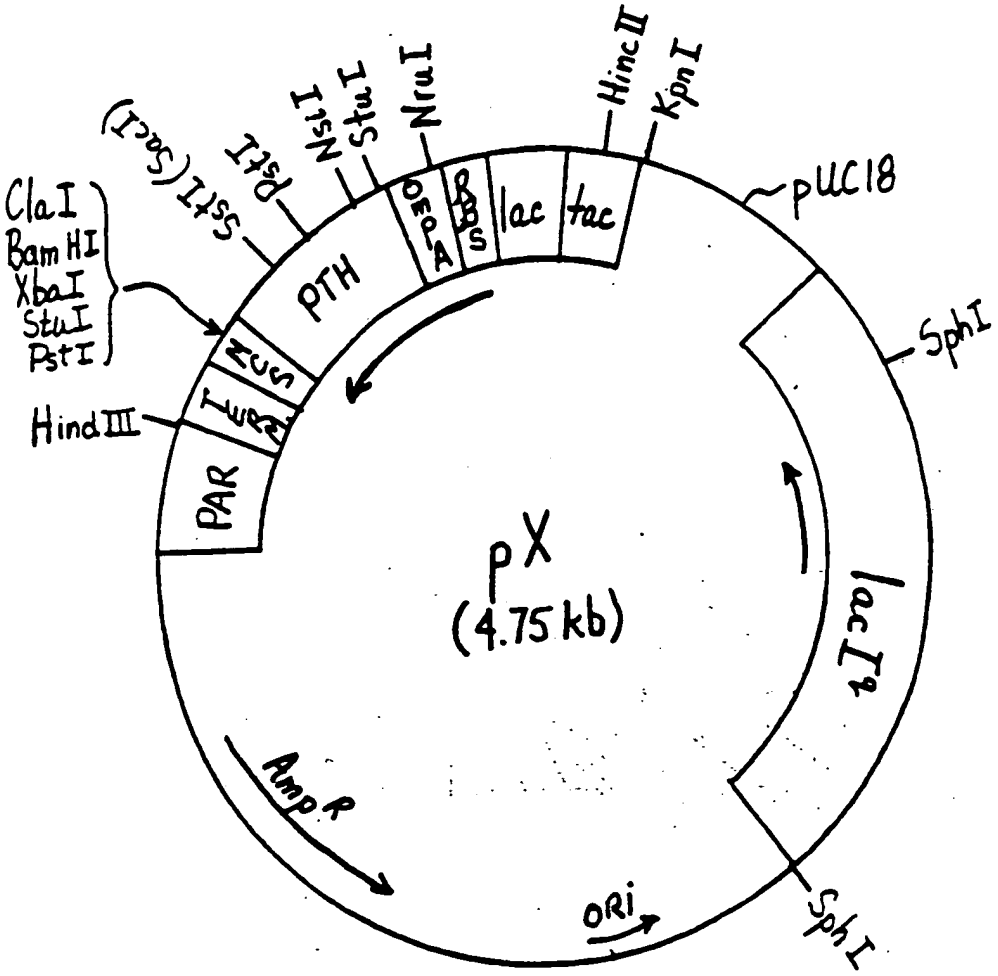
25. A method for producing a parathyroid hormone variant having substantial PTH activity and a reduced sensitivity to oxidation, which comprises the step of culturing a cellular host as defined in claim 22, claim 23 or claim 24.

26. A pharmaceutical composition comprising a pharmaceutically acceptable carrier and a parathyroid hormone variant as defined in any one of claims 1-17.

27. A pharmaceutical composition according to claim 26, wherein the variant is [Leu^{8}]hPTH.

28. A pharmaceutical composition according to claim 26, wherein the variant is [Leu^{18}]hPTH.

29. A pharmaceutical composition according to claim 26, wherein the variant is [$Leu^{8}Leu^{18}$]hPTH.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BamHI
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StuI
PstI
SstI (SacI)
PstI
NsiI
StuI
NruI
HincII
KpnI
pUC18
PTH
OEPA
RBS
lac
tac
MCS
T1
T2
HindIII
PAR
pX
(4.75 kb)
SphI
lacIq
Amp R
ORi
SphI

Figure 1

```
                1                                  10
                SerValSerGluIleGlnLeu Met HisAsnLeuGlyLysHisLeuAsnSer Met
ompA signal..TCTGTGAGTGAAATACAGCTT ATG CATAACCTGGGAAAACATCTGAACTCG ATG

    20                  30                  40
GluArgValGluTrpLeuArgLysLysLeuGlnAspValHisAsnPheValAlaLeuGlyAlaProLeuAla
GAGAGAGTAGAATGGCTGCGTAAGAAGCTGCAGGATGTGCACAATTTTGTTGCCCTTGGAGCTCCTCTAGCT

                50                  60
ProArgAspAlaGlySerGlnArgProArgLysLysGluAspAsnValLeuValGluSerHisGluLysSer
CCCAGAGATGCTGGTTCCCAGAGGCCCCGAAAAAAGGAAGACAATGTCTTGGTTGAGAGCCATGAAAAAAGT

        70                  80
LeuGlyGluAlaAspLysAlaAspValAsnValLeuThrLysAlaLysSerGln
CTTGGAGAGGCAGACAAAGCTGATGTGAATGTATTAACTAAAGCTAAATCCCAG...cloning
                                                          site/stop
                                                          codons...
```

FIGURE 2

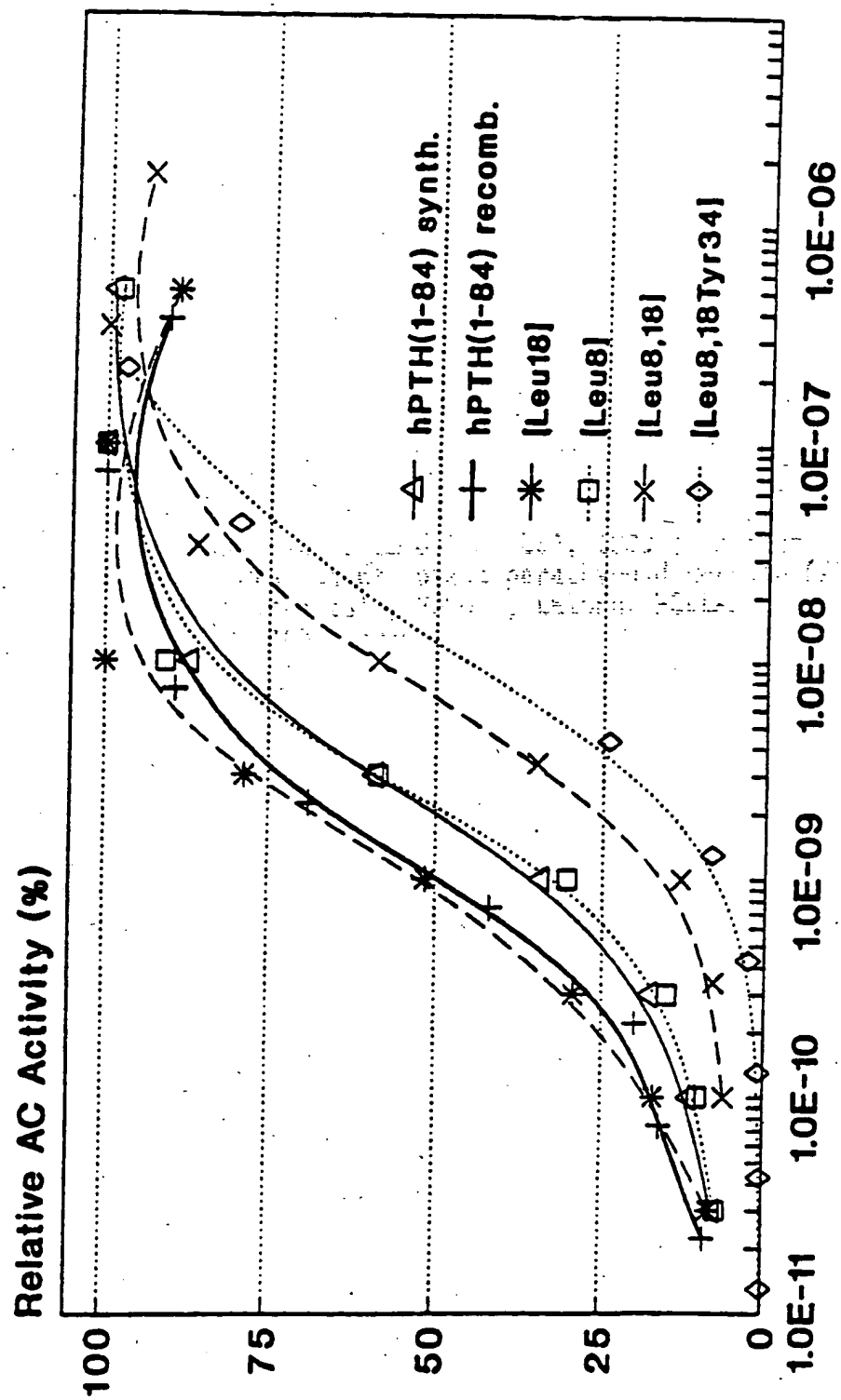

Figure 3 Relative adenylate cyclase activities (mock oxidized)

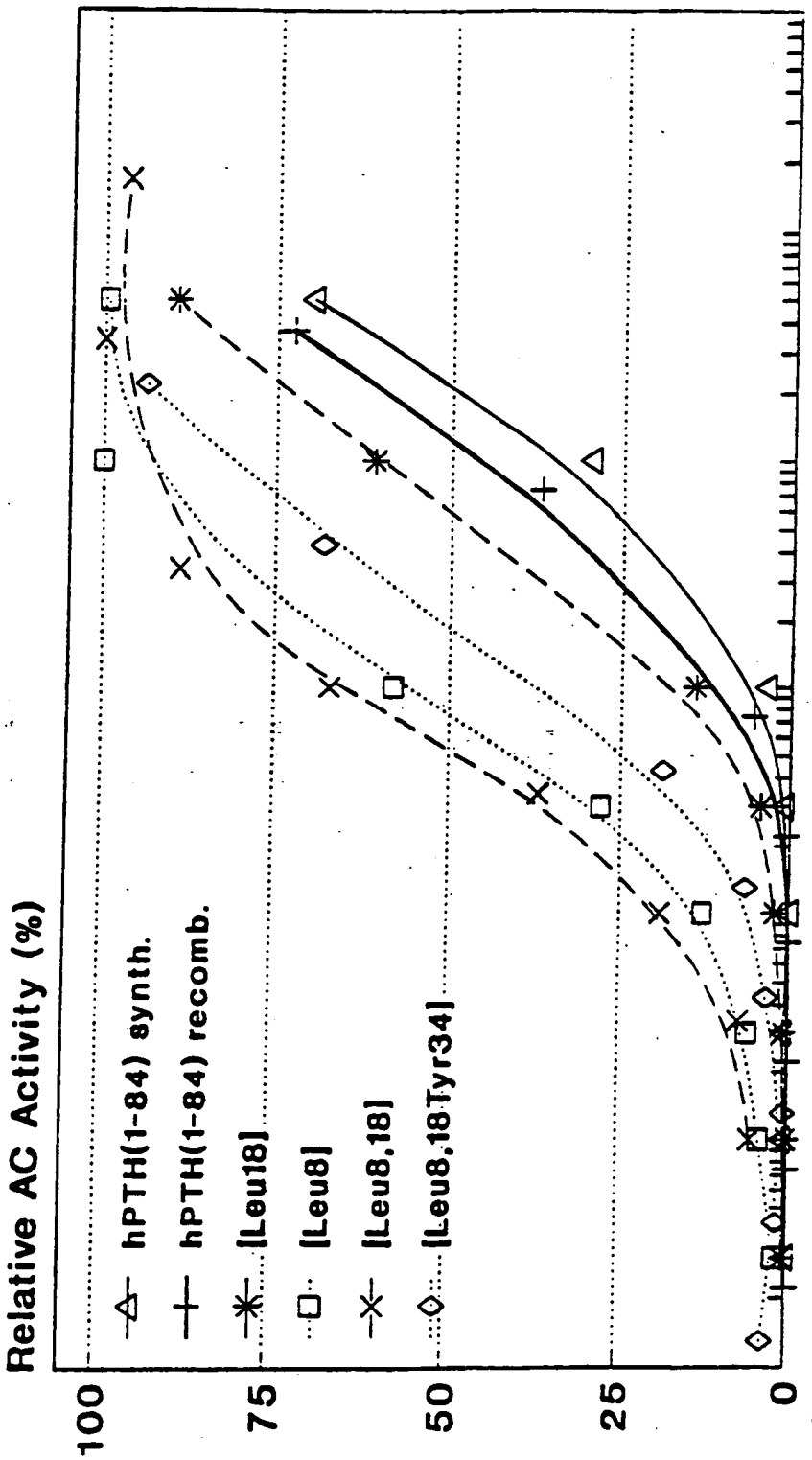

Figure 4 Relative adenylate cyclase activities (oxidized)

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C07K7/10; A61K37/24; C12N15/16

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C07K ; A61K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	EP,A,0 301 485 (GESELLSCHAFT FUR BIOTECHNOLOGISCHE FORSCHUNG) 1 February 1989 * Col. 5, l. 7-13 *	1,10-13, 15, 17-19, 22-29
X	EDITOR: RIVIER ET AL // AUTHOR: CHOREV ET AL 'Proceedings of the 11th American Peptide Symposium (La Jolla, USA, 1989) // Second generation of potent parathyroid hormone (PTH) antagonists' , ESCOM , LEIDEN, HOLLAND * P. 163, Table 1 *	1,10,14, 17-20,26
Y	BIOCHEMISTRY vol. 13, 1974, WASHINGTON pages 1994 - 1999; SAUER ET AL: 'The amino acid sequence of porcine parathyroid hormone' * P. 1998 (Discussion) *	1-19, 26-29

-/--

* Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

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"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
13 MARCH 1992	23.03.92
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	KORSNER S.E.

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	
Y	JOURNAL OF CLINICAL INVESTIGATION vol. 80, 1987, NEW YORK pages 1803 - 1807; STREWLER ET AL: 'Parathyroid hormonelike protein from human renal carcinoma cells' * P. 1806, Col 1 *	1-19, 26-29
Y	EP,A,0 260 350 (CETUS CORPORATION) 23 March 1988 * P. 2-3 (Introduction) * * Claims 1-3 *	1-19, 26-29

Form PCT/ISA/210 (extra sheet) (January 1985)

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. CA 9100451 SA 54568

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 13/03/92
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-0301485	01-02-89	DE-A- 3725320	09-02-89
		JP-A- 2138980	28-05-90
EP-A-0260350	23-03-88	None	